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Ploszaj et al., Amino acids (Austria), 2000, 19 (2), p483-96.

Stefanelli et al. Biochemical journal (England) May 1, 2000, 347 Pt. 3, p875-80.

Sakagami et al. Anticancer Research (Greece), Jan-Feb. 2000, 20 (1A), p265-70.

4 Ray et al., American journal of physiology, Cell physiology (US), Mar. 2000, 278 (3), pC480-9.

Bock et al. Radiation research (US), Dec. 1999, 152 (6), p604-10.

Dai et al. Cancer research (US), Oct. 1, 1999, 59 (19), p4944-54.

Bratton et al. Jo. of biological chemistry (US), Oct. 1, 1999, 274 (40), p28113-20.

Palyi et al. Anti-cancer drugs (England), Jan 1999, 10 (10), p103-11.

Li et al. Am. journal of physiology, April 1999, 276 (4 Pt. 1), pC946-54.

Ray et al. Am. journal of physiology, Mar. 1999, 276 (3 Pt. 1) pC684-91.

Das et al. Oncology Research (US), 1997, 9 (11-12), p565-72.

Monti et al.. Life Sciences (England), 1998, 62 (9), p799-806.

Lin et al., Experimental cell research, (US), Nov. 25, 1997.

Tome et al. biochemical Journal (England) Dec. 15, 1997, 328 (Pt. 3), p847-54.

Hu et al., Biochemical journal (England), Nov. 15, 1997, 328 (Pt. 1), p307-16.

Tome et al. biological signals (Switzerland), May -Jun 1997, 6 (3), p150-6.

Taguchi et al., Cell biochemistry and function (England), Mar 2001, 19 (1), p19-26.

Camon et al. neurotoxicology (US), Fall 1994, 15 (3), p759-63.

Shinki et al., Gastroenterology (US), Jan 1991, 100 (1), p113-22.

Heston et al. Prostrate (US), 1982, 3 (4), p383-9

Stefanelli et al, biochemical journal (England), Apr. 1, 2001. 355 (pt. 1), p199-206.

Lopez et al., biocell: official journal of the sociedades latinoamericanas de microscopia electronica... et. al. 9Argentina), Dec. 1999, 23 (3), p223-8.

Schipper et al. seminars in cancer biology (US), feb. 2000, 10 (1), p55-68.

Nilsson et al., biochemical journal (England) Mar. 15, 2000, 346 Pt. 3, p699-704.

giuseppina monti m. et al., biochemical and biophysical research commun. (US), Apr. 13, 1999, 257 (2), p460-5.

ratasirayakorn et al, j. of periodontology feb. 1999, 70 (2), p179-84

stabellini et al., Experimental and molecular pathology (US), 1997, 64 (3), p147-55.

Sparapani et al, experimental neurology (US), nov. 1997, 148 (1), p157-66..

Dhalluin et al., carcinogenesis (Eng.), Nov. 1997, 18 (11), p2217-23.

Polyamine depletion delays apoptosis of rat intestinal epithelial cells

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Ray, Ramesh M., Mary Jane Viar, Qing Yuan, and L. R. Johnson. Polyamine depletion delays apoptosis of rat intestinal epithelial cells. *Am. J. Physiol. Cell Physiol.* 278: C480–C489, 2000.—The polyamines spermidine, spermine, and their precursor putrescine are essential for cell growth and the regulation of the cell cycle. Recent studies suggest that excessive accumulation of polyamines favors either malignant transformation or apoptosis, depending on the cell type and the stimulus. This study examines the involvement of polyamines in the induction of apoptosis by the DNA topoisomerase I inhibitor, camptothecin. In IEC-6 cells, camptothecin induced apoptosis within 6 h, accompanied by detachment of cells. Detached cells showed DNA laddering and caspase 3 induction, characteristic features of apoptosis. Depletion of putrescine, spermidine, and spermine by DL- α -difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase (ODC) that is the first rate-limiting enzyme for polyamine biosynthesis, decreased the apoptotic index. Delayed apoptosis was accompanied by a decrease in caspase 3 activity in polyamine-depleted cells. Addition of putrescine restored the induction of apoptosis as indicated by an increase in the number of detached cells and caspase 3 activity. Polyamine depletion did not change the level of caspase 3 protein. Inhibition of S-adenosylmethionine decarboxylase by a specific inhibitor [diethylglyoxal bis-(guanyldrazone); DEGBG] led to depletion of spermidine and spermine with a significant accumulation of putrescine and induction of ODC. The DEGBG-treated cells showed an increase in apoptosis, suggesting the importance of putrescine in the apoptotic process. Addition of putrescine to DFMO-treated cell extracts did not increase caspase 3 activity. The above results indicate that polyamine depletion delays the onset of apoptosis in IEC-6 cells and confers protection against DNA damaging agents, suggesting that polyamines might be involved in the caspase activating signal cascade.

caspases; DL- α -difluoromethylornithine; putrescine; spermidine; spermine

CELLS REQUIRE OPTIMAL LEVELS of the polyamines putrescine, spermidine, and spermine for growth and differentiation and are therefore equipped with intricate mechanisms for the control of polyamine levels (10, 19, 29, 39, 40, 62). Any cell that becomes polyamine-deficient is severely limited in its ability to grow and proliferate.

Our laboratory has studied the role of polyamines in the growth and repair of the gastrointestinal mucosa and has examined these processes in both rats and in cultured intestinal IEC-6 epithelial cells, a nontransformed line derived from fetal rat crypt cells (45). In IEC-6 cells, inhibition of ornithine decarboxylase (ODC), which catalyzes the first rate-limiting step in polyamine biosynthesis by α -difluoromethylornithine (DFMO), depletes polyamines, inhibits growth, and prevents normal repair of mucosal stress ulcers (31, 59, 60). Recently, we demonstrated that depletion of polyamines leads to cell cycle arrest but does not induce apoptosis in IEC-6 cells (47). There is also evidence suggesting that polyamines play a role in apoptotic cell death (16, 38, 42, 44, 64). Tobias and Kahana (57) and Pouline et al. (44) reported that excessive accumulation of polyamines in ODC-overproducing cells induced apoptosis (44, 57).

Apoptosis, or programmed cell death, is the result of biochemical events carried out by a family of cysteine proteases called caspases. Caspases were implicated in apoptosis with the discovery that CED-3, the product of a gene required for cell death in the nematode *Caenorhabditis elegans*, is related to mammalian interleukin-1 β -converting enzyme (ICE or caspase 1) (56, 65, 66). Caspases share similarities in amino acid sequence, structure, and substrate specificity (37). They are all expressed as proenzymes (30–50 kDa) that contain three domains: an NH₂-terminal domain, a large subunit (~20 kDa), and a small subunit (~10 kDa). Caspase activation requires proteolytic cleavage between the domains and the formation of heterodimers. The mechanisms and series of events that cause apoptosis are becoming clearer. Degradation of nuclear DNA at internucleosomal sites occurs during the final stages of apoptosis, but is not essential, because apoptosis occurs in enucleated cells (58). On the other hand, cell signaling involving Src homology 2 (SH2) domains and proteolysis may respectively cue the "initiation" and "execution" phases of apoptosis (12). In *Xenopus* extracts, SH2 fusion proteins and related synthetic peptides inhibited the initiation of programmed cell death (PCD). In other work, recombinant caspase expression increased PCD (33), whereas protease inhibition prevented it (26, 50). This death-promoting action of caspases and other proteases is associated with the cleavage of specific proteins. The initiation and execution phases of PCD may be connected in that apoptotic proteases target specific pro-

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tein substrates essential to signal transduction. For example, in one cell system, serine proteases attacked PITSLRE (conserved PSTAIRE box region of cdc2 p34) kinase, generating a truncated apoptosis-inducing isoform (27). Similarly, cysteine proteases belonging to the CED-3/ICE family cleave the murine double minute (MDM1) oncoprotein, which normally downregulates p53 (11). This increases p53 and leads to apoptosis in this system.

Apoptosis is vitally important for development and maintenance of tissue homeostasis and is a tightly regulated process (63). Intestinal cells derived from stem cells at the base of the intestinal crypts proliferate and migrate along the crypt-villus axis until they reach the lumen. At the luminal surface, they lose anchorage, detach, and undergo apoptosis (14, 53). Expression of the Bcl2/Bax family of proteins, cell to cell contact, cell to extracellular matrix attachment, integrin expression, cytokines, environmental factors, and chemical agents may contribute to the induction of apoptosis at the luminal surface (2, 3, 20, 36, 52). Detachment-induced cell death is a recognized form of apoptosis in freshly isolated human intestinal epithelial cells (17, 54) and other anchorage-dependent cell types, such as bronchial epithelial cells, mammary epithelial cells, renal epithelial cells, and endothelial cells (1, 4, 13, 33, 48). A nontumorigenic line of rat intestinal epithelial cells (IEC-18) normally died within 48–72 h when cultured as multicellular spheroids on a nonadhesive surface (46). Yet, mutant c-H-ras expression in these cells inhibited PCD. Finally, inducible oncogenic ras has been shown in interleukin-3-dependent hematopoietic cells to upregulate "survival" proteins, bcl-2 and bcl-xl, suppressing PCD (23). Although it is not yet determined that Ras activation directly promotes the survival of intestinal epithelial cells through either phosphatidylinositol 3-kinase (PI3K) or the Raf pathway, in c-myc-induced fibroblast apoptosis, ras can have either an anti-apoptotic or a proapoptotic action depending on whether PI3K or Raf kinase pathways are respectively activated (22, 51).

Based on our previous work showing that polyamine depletion upregulated p53 without causing apoptosis, and because apoptosis is an important process in the maintenance of tissue homeostasis of gut epithelium, we investigated the role of polyamines in camptothecin-induced apoptosis in IEC-6 cells.

MATERIALS AND METHODS

Materials. Medium and other cell culture reagents were obtained from GIBCO BRL (Grand Island, NY). Fetal bovine serum (FBS), dialyzed FBS (dFBS; 10,000 mol wt cutoff), and camptothecin were purchased from Sigma (St. Louis, MO). Enhanced chemiluminescence Western blot detection system was purchased from DuPont-NEN (Boston, MA). DFMO was a gift from Merrell Dow (Cincinnati, OH), and diethylglyoxal bis-(guanyldihydrazone) (DEGBG) was synthesized for our laboratory by Dr. Patrick J. Rodrigues and Dr. Mervyn Israel in the Department of Pharmacology at the University of Tennessee, Memphis. Anti-caspase 3 (CPP 32) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TACS apoptotic DNA laddering kit was purchased from Trevigen

(Gaithersburg, MD). Colorimetric caspase assay reagents were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). The cell death detection ELISA plus kit was purchased from Roche Diagnostics (Indianapolis, IN). The IEC-6 cell line (ATCC CRL 1592) was obtained from American Type Culture Collection (Rockville, MD) at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (45). The cells were nontumorigenic and retained the undifferentiated character of epithelial stem cells. All other chemicals were of the highest purity commercially available.

Cell culture. IEC-6 cell stock was maintained in T-150 flasks in a humidified 37°C incubator in an atmosphere of 90% air-10% CO₂. The medium consisted of DMEM with 5% heat-inactivated FBS, 10 µg insulin, and 50 µg gentamicin sulfate/ml. The stock was passaged weekly at 1:10, fed three times per week, and passages 15–20 were used. For the experiments, the cells were taken up with 0.05% trypsin plus 0.53 mM EDTA in Hanks' balanced salt solution (HBSS) without calcium and magnesium and counted by hemocytometer.

Apoptosis studies. The cells were plated (day 0) at a density of 6.25×10^4 cells/cm² in T-150 flasks in DMEM/dFBS with or without the treatment compound(s) and with triplicate samples for each group. Cells were fed on day 2. On day 3, medium was removed and replaced with serum-free medium. On day 4, camptothecin (20 µM in DMSO) or tumor necrosis factor-α (TNF-α; 20 ng/ml with or without 25 µg/ml cycloheximide) was added into the serum-free medium for 3–18 h with the appropriate vehicle added to controls.

Cell number. Cell counts were determined separately for floating and attached cells. Floating cells were poured into a 25-ml tube and the monolayer was washed once with HBSS without calcium and magnesium. This wash was then combined into the tube with the floating cells. Attached cells were taken up with 0.05% trypsin plus 0.53 mM EDTA, followed by one wash of the flask with DMEM/5% FBS. Cells were counted on a model Z_F Coulter counter. Although flasks contained approximately equal numbers of cells, floating cells were expressed as a percentage of the total cell count obtained by combining the number of floating and attached cells.

Caspase activity. After treatment with camptothecin (or vehicle) on day 4, floating cells were poured off to be counted and combined with one wash with cold Dulbecco's phosphate buffer saline (DPBS). The attached cells were then harvested for determination of caspase activity. Briefly, 10 ml of DPBS was added to the flask, and the monolayer was scraped and collected into a 25-ml tube. The flask was washed once with 10 ml of DPBS and combined into the 25-ml tube. The cells were pelleted by centrifugation at 800 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml of cold DPBS and transferred into a microcentrifuge tube. The cells were pelleted by centrifugation at 10,000 *g* at 4°C for 10 min. The supernatant was discarded and the cells were lysed in 100 µl of ice-cold cell lysis buffer (50 mM HEPES, pH 7.4, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1% Nonidet P-40). The assay for caspase activity was carried out in a 96-well plate. Into each well was placed 20 µl of cell lysate, 70 µl of assay buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 100 mM NaCl, 10 mM DTT, and 1 mM EDTA), and 10 µl of caspase 3 colorimetric substrate (2 mM DEVD-pNA prepared in assay buffer), a caspase specific peptide that is conjugated to the chromogen *p*-nitroanilide (pNA). The 96-well plate was incubated at 37°C for 2 h, during which time the caspase in the sample was allowed to cleave the

chromophore p-NA from the substrate molecule. Absorbance readings at 405 nm were made after the 2 h incubation, with the caspase activity being directly proportional to the color reaction. Protein was determined for each sample using the bicinchoninic acid method (Pierce, Rockford, IL) and a standard curve for pNA was constructed. Caspase activity was expressed as picomole of pNA released per milligram of protein per minute.

DNA fragmentation. After treatment with camptothecin (or vehicle), floating cells were poured off and combined with one DPBS wash of the monolayer. Cells were pelleted by centrifugation at 800 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in 100 μ l of resuspension buffer. The attached cells were collected for DNA fragmentation by scraping in DPBS and combining with one wash of the flask with DPBS. The cells were pelleted at 800 *g* for 5 min, the supernatant was discarded, and the pellet was resuspended in 100 μ l of resuspension buffer. The nucleosomal fragmentation assay was carried out by isolating DNA from the cells using the TACS apoptotic DNA laddering kit and analyzing the labeled DNA by agarose gel electrophoresis according to the manufacturer's instructions.

Quantitative DNA fragmentation ELISA. Cells were grown in six-well culture plates. After treatment with camptothecin (or vehicle), floating cells were discarded and the attached cells were collected by trypsinization. Cells were pelleted by centrifugation at 800 *g* for 5 min, washed once with DPBS, and resuspended in 3 ml DPBS. One milliliter of the cell suspension was taken for protein determination and the remainder were used for the DNA fragmentation ELISA. Cells were processed according to instructions provided in the kit. Briefly, cells were lysed and centrifuged to remove the nuclei. An aliquot of the nuclei-free supernatant was placed into the streptavidin-coated wells and incubated with anti-histone-biotin and anti-DNA peroxidase conjugate for 2 h at room temperature. After incubation, the sample was removed and the wells were washed three times with incubation buffer. After the final wash was removed, 100 μ l of the substrate 2,2'-azino-di[3-ethylbenzthiazolin-sulfonate] was placed in the wells for 20 min at room temperature. The absorbance was read at 405 nm using a plate reader. Results were expressed as absorbance at 405 nm/mg protein.

Western blot analysis. Total cell protein (50 μ g) from cell extracts prepared for caspase 3 assay was separated on 15% SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. Equal loading of protein was confirmed by staining the nitrocellulose membrane with Ponceau S. The membrane was then probed with an antibody directed against caspase 3. The immunocomplexes were visualized by the enhanced chemiluminescence detection system.

Polyamine levels. Intracellular polyamines were analyzed by HPLC as previously described (58). Briefly, cells were plated in 60-mm dishes at 6.25×10^4 cells/cm² and grown in DMEM/dFBS with or without DFMO or DEGBG. After washing the monolayer three times with ice-cold DPBS, we added 0.5 M perchloric acid and then froze the samples at -80°C until ready for extraction, dansylation, and HPLC. The standard curve encompassed the range of 0.31–10 μ M. Values that fell >25% below the curve were considered not detectable. Protein was determined by the Bradford method (5).

Statistics. Data are means \pm SE from representative experiments. All experiments were repeated three times ($n = 3$). Absence of error bars in Figs. 3, 4, 6, and 7 indicates that SE was too small to be seen as separate from the mean. The significance of the differences between means was determined by ANOVA and appropriate post hoc testing. Values of $P < 0.05$ were regarded as significant.

RESULTS

Induction of apoptosis by camptothecin. Recently, we reported that polyamine depletion increases levels of p53 and arrests the cell cycle in G1 phase but does not induce apoptosis in IEC-6 cells (47). This observation suggested the intriguing possibility that polyamines were involved in the apoptotic process in general. To examine the role of polyamines in the regulation of apoptosis in IEC-6 cells, we used a chemical agent, camptothecin (DNA topoisomerase I inhibitor), which is known to induce apoptosis in a wide variety of cells (21, 28, 49). Detachment-induced cell death has been reported in isolated intact intestinal crypts (17); therefore, we presumed that cells undergoing apoptosis would detach and that the extent of detachment could be used as an apoptotic index.

To establish the method of evaluation of apoptosis in IEC-6 cells, we treated confluent IEC-6 cells with camptothecin (20 μ g/ml) and monitored the detachment of cells from the dish surface. We observed a significant increase (8-fold) in the number of floating cells in the camptothecin treated group compared with the DMSO-treated (the vehicle for camptothecin) control group within 6 h of treatment (Fig. 1A). Detached and attached cells were harvested and examined for evidence of apoptosis by caspase 3 activity using DEVD-pNA as substrate and by DNA fragmentation. For this experiment, cells were treated with 20 μ M camptothecin for a longer period of time (16 h) to harvest enough floating cells for analysis. With camptothecin treatment, attached cells showed a sevenfold increase in caspase 3 activity (Fig. 1B) but did not show remarkable DNA fragmentation (Fig. 1C). Detached cells from both the control and the camptothecin-treated groups showed characteristic nucleosomal size DNA fragments and increased caspase 3 activity (Fig. 1B and C). Because the DNA fragmentation assay and caspase 3 activity indicated that detached cells were apoptotic, in subsequent experiments, caspase 3 activity was measured only in attached cells as a marker of the "initiation" phase of apoptosis. These results demonstrate the effectiveness of the DNA topoisomerase I inhibitor for the induction of apoptosis in IEC-6 cells.

Effect of polyamine depletion on the induction of apoptosis. To study the involvement of polyamines in camptothecin-induced apoptosis, IEC-6 cells were grown for 4 days in the presence or absence of DFMO, the highly specific inhibitor of ODC. Previous studies from this laboratory have shown that DFMO treatment depletes putrescine within 3 h, but that depletion of spermidine requires 24 h, and significant depletion (about 50%) of spermine requires 3 days (32).

We examined apoptosis 6 h after camptothecin treatment. Cells treated with DFMO (polyamine depleted) were protected against both DMSO (vehicle) and camptothecin-induced apoptosis. A decrease (of about 3-fold) in the number of floating cells was observed in polyamine-depleted cells compared with control cells treated with camptothecin (Fig. 2A). Short-term DFMO treatment along with camptothecin (6 h) did not show any

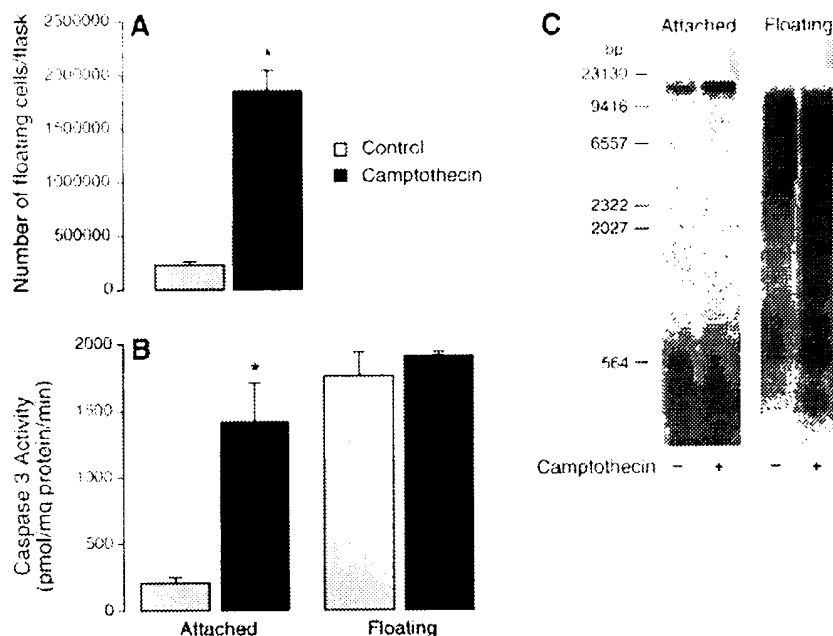


Fig. 1. Camptothecin induces apoptosis. Cells were grown for 3 days in DMEM/5% dialyzed fetal bovine serum (dFBS) and then serum deprived for 24 h before treatment with either DMSO (vehicle) or 20 μ M camptothecin for 6 h. **A**: number of floating cells. **B**: caspase 3 activity from attached and floating cells. **C**: DNA fragmentation analysis from attached and floating cells. * $P < 0.05$ compared with corresponding control value.

difference in the number of floating cells (data not shown). DFMO treatment also resulted in ~50% lower caspase 3 activity in attached cells compared with control (Fig. 2B).

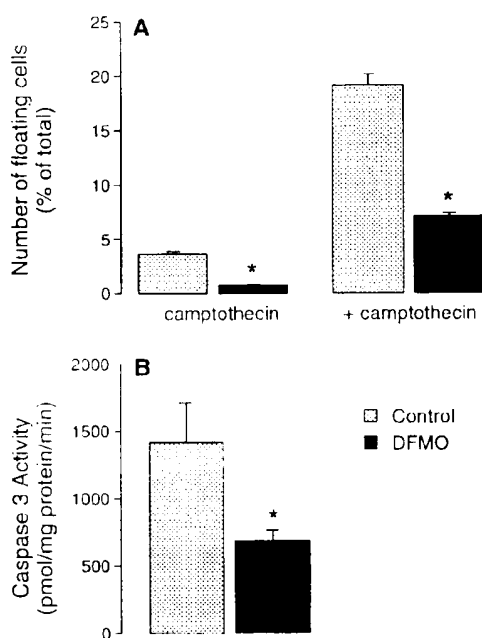


Fig. 2. Polyamine depletion inhibits camptothecin-induced apoptosis. Cells were grown for 3 days in DMEM/5% dFBS with or without 5 mM α -difluoromethylornithine (DFMO). Cells were serum deprived for 24 h before treatment with either DMSO (vehicle) or 20 μ M camptothecin for 6 h. Total cell counts were determined for control and DFMO treatment groups to express floating cell number as percent of total cell count. **A**: number of floating cells. **B**: caspase 3 activity from attached cells. * $P < 0.05$ compared with corresponding control value.

To gain insight to the apoptotic induction profile, we examined the time course of the induction of apoptosis by camptothecin in control and polyamine-depleted conditions. Results in Fig. 3A indicate that camptothecin-induced apoptosis (as judged by the number of floating cells) began within 3 h and increased progressively up to 18 h (from 6.2% at time 0 to 49.3% at 18 h) in control cells. Interestingly, the onset of apoptosis was delayed in DFMO-treated cells for up to 12 h (from 2.2% at time 0 to 9.3% at 12 h) and reached a maximum of 29.1% at 18 h. As mentioned previously, to understand early events in caspase induction, attached cells from the above experiment were collected for the determination of caspase 3 activity. Caspase 3 activity increased progressively with time in control cells. In contrast, DFMO-treated cells did not show remarkable increases in caspase 3 activity for up to 12 h. A significant increase in caspase 3 activity was observed only after 18 h of exposure to camptothecin (Fig. 3B). The above results clearly indicate that polyamine depletion delays the onset of apoptosis in IEC-6 cells, and suggest that intracellular polyamine levels may be critical factors in the regulation of spontaneous apoptosis in intestinal cell homeostasis.

We next determined whether the decrease in apoptosis seen after polyamine depletion could be prevented by exogenous polyamines. As shown in Fig. 4, DFMO again decreased the number of floating cells compared with control. Addition of exogenous 10 μ M putrescine along with DFMO restored the number of floating cells to control levels. DNA fragmentation was evident in floating cells irrespective of the treatment.

To determine the specificity of the effect of polyamine depletion on apoptosis, we used TNF- α , a receptor-mediated physiological inducer of apoptosis, along with cycloheximide. Neither TNF- α (20 ng/ml) nor cyclohexi-

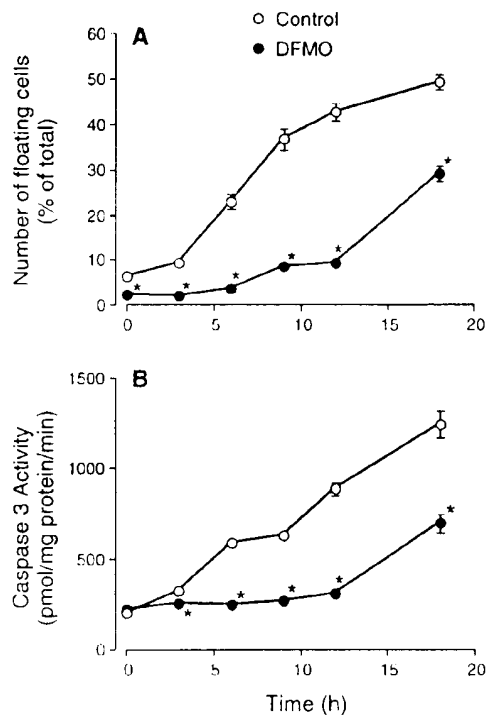


Fig. 3. Time-dependent induction of apoptosis by camptothecin is delayed by polyamine depletion. Cells were grown for 3 days in DMEM/5% dFBS with or without 5 mM DFMO. Cells were serum deprived for 24 h before treatment with 20 μ M camptothecin for 6 h. Total cell counts were determined for control and DFMO treatment groups to express floating cell number as percent of total cell count. A: number of floating cells. B: caspase 3 activity from attached cells. * P < 0.05 compared with corresponding control value.

mide (25 μ g/ml) alone induced apoptosis in IEC-6 cells (data not shown). However, treatment of the cells with TNF- α plus cycloheximide resulted in a significant induction of apoptosis in control cells (Fig. 5A). As was the case with camptothecin, polyamine depletion protected against apoptosis induced by TNF- α plus cycloheximide. Addition of exogenous putrescine to DFMO-treated cells resulted in control levels of apoptosis.

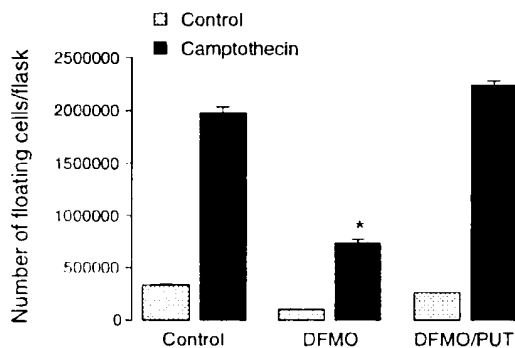


Fig. 4. Exogenous putrescine restores camptothecin-induced apoptosis. Cells were grown for 3 days in DMEM/5% dFBS with or without 5 mM DFMO and 10 μ M putrescine (PUT). Cells were serum deprived for 24 h before treatment with either DMSO (vehicle) or 20 μ M camptothecin for 6 h. * P < 0.05 compared with corresponding control value.

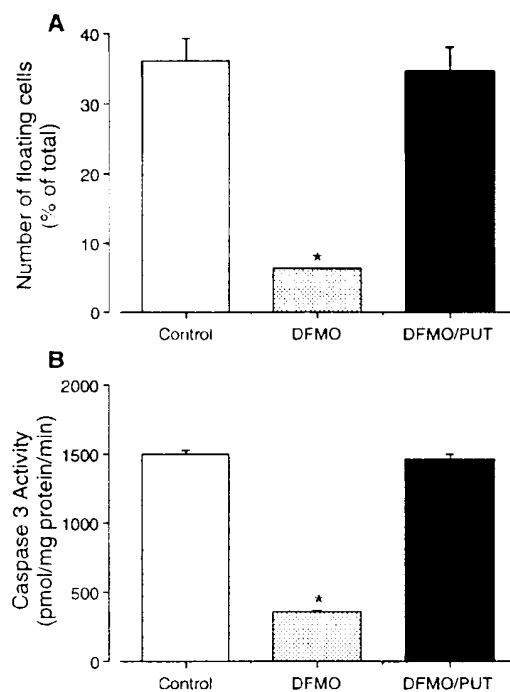


Fig. 5. Inhibition of TNF- α induced apoptosis by polyamine depletion is restored by exogenous putrescine. Cells were grown for 3 days in DMEM/5% dFBS with or without 5 mM DFMO and 10 μ M putrescine. Cells were serum deprived for 24 h before treatment with 20 ng/ml TNF- α plus 25 μ g/ml cycloheximide for 6 h. Total cell counts were determined for control, DFMO, and DFMO plus putrescine treatment groups to express floating cell number as percent of total cell count. A: number of floating cells. B: caspase 3 activity from attached cells. * P < 0.05 compared with corresponding control value.

Caspase 3 activity in the attached cells from this experiment showed a pattern that was identical to that seen with camptothecin. Figure 5B shows that TNF- α and cycloheximide significantly increased caspase 3 activity. This increase was largely prevented by DFMO and restored by the addition of exogenous putrescine in the presence of DFMO.

Intracellular polyamine levels can be altered by inhibiting either ODC or S-adenosylmethionine decarboxylase (SAM-DC) activities by DFMO or DEGBG, respectively. DEGBG blocks the conversion of putrescine to spermidine and spermine resulting in high intracellular putrescine and low spermidine and spermine levels. IEC-6 cells were grown in the presence and absence of DEGBG, and intracellular polyamine levels were determined. Results in Fig. 6A show that DEGBG significantly increased the putrescine level and significantly decreased the levels of spermidine (Fig. 6B) and spermine (Fig. 6C) over 4 days of treatment. These results indicate that inhibition of SAM-DC leads to an accumulation of putrescine with concomitant depletion of spermidine and spermine. Because intracellular putrescine is rapidly converted to spermidine and spermine, results in Fig. 4 do not indicate which of the polyamines may be involved in the apoptotic process. Therefore, putrescine accumulation and ODC induction by DEGBG proved to be an important tool for

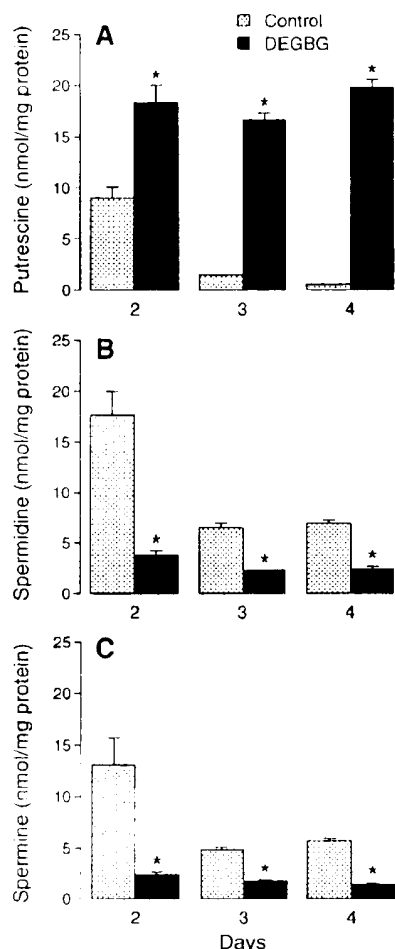


Fig. 6. S-adenosylmethionine decarboxylase (SAM-DC) inhibition results in accumulation of putrescine (A) and depletion of spermidine (B) and spermine (C). Cells were grown in DMEM/5% dFBS with or without 1 mM diethylglyoxal bis-(guanyldrazone) (DEGBG). Cells were serum deprived for 24 h before harvesting for polyamine analysis. Polyamine concentrations were measured by HPLC and expressed as nanomoles per milligram of protein. * $P < 0.05$ compared with corresponding control value.

which to study the role of putrescine in apoptosis. In the following experiment, percentages of floating cells were calculated based on total cell number for each treatment group. Figure 7A shows that DFMO led to a progressive decrease in the percentage of floating cells over days 1, 2, and 4 (19.2, 12.4, and 2.2%) in response to camptothecin. Compared with control (18.8%), DFMO treatment for only 1 day, which results in putrescine depletion, but not spermidine or spermine depletion, did not affect the percentage of floating cells. In contrast to this, DEGBG treatment significantly increased the percentage of floating cells on days 1, 2, and 4 (26.6, 26.9, and 23.3%) compared with DFMO-treated as well as with control cells. Caspase 3 activity followed the same pattern as that for the number of floating cells (Fig. 7B). These results show that accumulation of putrescine is associated with the induction of apoptosis,

whereas depletion of spermidine and spermine leads to the inhibition of apoptosis in IEC-6 cells.

To confirm the above results, we examined the effects of the polyamine modulators (DFMO and DEGBG) on another index of apoptosis, quantitative DNA fragmentation by an ELISA method. Treatment with 20 μ M camptothecin for 6 h resulted in a significant increase in DNA fragmentation in control cells (Fig. 8). The extent of DNA fragmentation was inhibited by polyamine depletion and was significantly restored by exogenous putrescine. Inhibition of SAM-DC activity by DEGBG did not significantly affect the degree of DNA fragmentation. These results for DNA fragmentation are in agreement with both the caspase 3 activity and floating cell number indices of apoptosis.

The above results suggest that polyamine depletion either decreases the level of caspase 3 protein or that putrescine may be required for the activation of caspase 3 or for the signal transduction cascade involved in the induction of apoptosis. Figure 9 shows the levels of procaspase 3 (32 kDa protein) in the attached cells grown in control, DFMO, and DFMO/putrescine conditions. Levels of procaspase 3 were not significantly different in polyamine-depleted cells vs. controls or

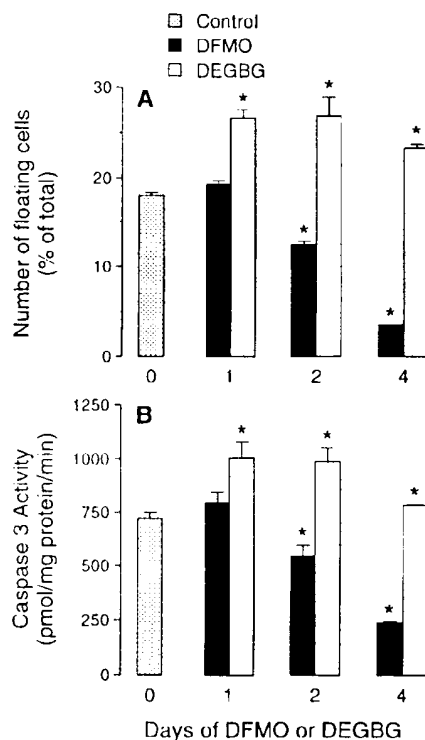


Fig. 7. Modulation of polyamine levels by inhibition of ornithine decarboxylase and SAM-DC affects apoptotic indices. Cells were grown in DMEM/5% dFBS for 4 days. Cells were treated with either 5 mM DFMO or 1 mM DEGBG for indicated time during 4-day growth period. Cells were serum deprived for 24 h before treatment with 20 μ M camptothecin for 6 h. Total cell counts were determined for control, DFMO, and DEGBG treatment groups to express floating cell number as percent of total cell count. A: number of floating cells. B: caspase 3 activity from attached cells. * $P < 0.05$ compared with corresponding control value.

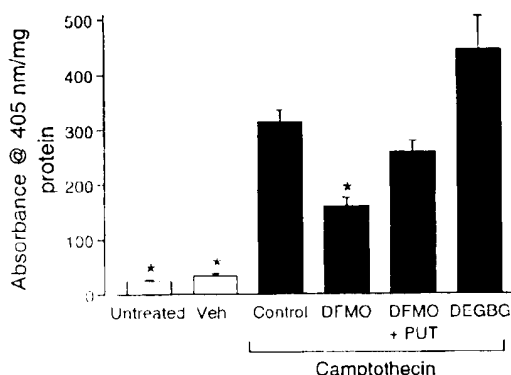


Fig. 8. Polyamines influence DNA fragmentation after induction of apoptosis by camptothecin. Cells were grown for 3 days in DMEM/5% dFBS with or without 5 mM DFMO + PUT or 1 mM DEGBG. Cells were serum deprived for 24 h before treatment with 20 μ M camptothecin for 6 h. DNA fragmentation was assayed by ELISA and was quantitated based on absorbance at 405 nm. Statistical significance was determined based on comparison of all groups with camptothecin-treated control group. * $P < 0.05$ compared with corresponding control value.

those treated with DFMO plus putrescine. To determine whether putrescine directly affected caspase 3 activity, we used cell extracts from DFMO-treated cells which were low in caspase 3 activity and added putrescine into the reaction mixture during the caspase 3 activity assay. Increasing putrescine concentrations up to 5 μ M had no effect on caspase activity (data not shown).

DISCUSSION

Apoptosis is required for all multicellular organisms to achieve and maintain homeostasis of cell numbers within their tissues. Increased apoptosis produces degenerative diseases of the central nervous system or immunodeficiencies, whereas failure to undergo apoptosis results in developmental abnormalities and cancer (20, 63). The gastrointestinal tract is no exception, and the normal regulation of apoptosis is crucial to preventing hyperplasia and malignancies. In the small intestine, spontaneous apoptosis during development is necessary to achieve the optimal number of stem cells. Thus understanding the mechanisms that influence and control apoptosis in the intestinal epithelium has considerable clinical as well as physiological relevance. Previous studies from our laboratory have established the involvement of polyamines in the regulation of intestinal epithelial cell proliferation and migration (31, 32, 60). We have shown that polyamine depletion of

IEC-6 cells increases levels of p53 and p21 and leads to cell cycle arrest (47). Chen et al. (7) have shown that p53 and the extent of DNA damage, among other variables, can lead either to cell cycle arrest or apoptosis. In fact, p53 may induce apoptosis in some cells and cell cycle arrest in others in response to the same stimulus. We found no evidence of increased apoptosis in IEC-6 cells undergoing cell cycle arrest in response to DFMO.

These findings have led us in the present study to examine the influence of polyamine levels on the induction of apoptosis in IEC-6 cells by an agent that causes DNA damage. Camptothecin, an inhibitor of DNA topoisomerase I, has been used widely to induce apoptosis under experimental conditions and is in phase III clinical trials for the treatment of colon cancer (8, 9). Camptothecin proved to be an effective apoptotic agent in our IEC-6 cell model. Camptothecin led to detachment of cells from the substratum as evidenced by floating cells in the medium. Floating cells exhibited nucleosomal DNA fragmentation characteristic of apoptosis (Fig. 1A and B). The induction of caspase activity, considered to be an early event in the apoptotic pathway, was measured in attached cells as well as in floating cells. Floating cells, irrespective of the presence or absence of camptothecin, showed caspase activity as well as DNA fragmentation (Fig. 1). In contrast, attached cells treated with camptothecin had very high levels of caspase 3 activity compared with control cells. The above results indicate that detachment of cells can be used as an index for detection of apoptosis, and that camptothecin induces caspase 3 activity in IEC-6 cells. Although cellular substrates for the caspase family of proteases involved in apoptosis are not well understood, caspase 3 has been shown to cleave focal adhesion kinase, gelsolin, and actin (15, 24, 30). This suggests that detachment of cells during induction of apoptosis by camptothecin may be due to the cleavage of cytoskeletal proteins by caspases causing disruption of the cytoskeleton and focal adhesion complexes.

Polyamines are critical for optimal cell growth, and the polyamine pool shows only modest changes during the cell cycle (6, 10, 40). Results depicted in Figs. 2 and 3 clearly indicate that a certain level of intracellular polyamines is necessary for apoptosis. Four days' treatment with DFMO and subsequent polyamine depletion delayed the apoptotic response to camptothecin by at least 12 h. Cells grown for 4 days in the presence of DFMO, and exogenous putrescine had apoptotic responses indistinguishable from control cells (Figs. 4 and 5). Thus there is no doubt that the effects of DFMO are produced by polyamine depletion, and that they are due specifically to the absence of physiologically active polyamines.

The questions of which polyamines and what intracellular levels must be present for normal effects are interesting ones. DFMO and the subsequent inhibition of ODC cause time-dependent decreases in intracellular putrescine, followed by spermidine and spermine. In IEC-6 cells, putrescine disappears by 3 h, spermidine by 24 h, and spermine remains at levels equal to

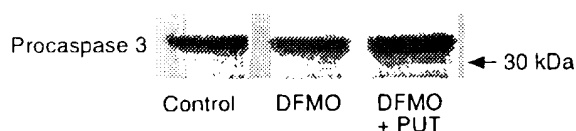


Fig. 9. Polyamine depletion does not alter procaspase 3 protein level. Cells were grown for 3 days in DMEM/5% dFBS with or without 5 mM DFMO and 10 μ M putrescine. Cells were serum deprived for 24 h before harvesting cells for preparation of cell lysate for Western immunoblotting.

around 40% of control even after 3 days' incubation with DFMO (32). As shown in Fig. 7, there was a significant decrease in apoptosis after 2 days' treatment with DFMO. At this time, half the intracellular spermine is still present, but because of their strong positive charge, most polyamines probably exist bound to macromolecules like proteins and DNA. It may well be that the only active polyamines, in terms of regulating physiological processes, are those that are free. Thus the free polyamine pool may decrease within a few hours after the inhibition of putrescine production.

When exogenous putrescine is supplied to cells incubated with DFMO, it restores cell growth, migration, and apoptosis to normal. But these effects are not caused necessarily by putrescine, for it is rapidly converted to spermidine and then to spermine. Putrescine depletion by itself (DFMO treatment for 1 day) did not affect the apoptotic index or caspase induction (Fig. 7). However, with spermidine and spermine depletion (DFMO treatment for 2 to 4 days), apoptosis was significantly decreased. On the other hand, putrescine accumulation after DEGBG treatment led to a remarkable increase in cell detachment and caspase activity compared with DFMO and control cells. Differences in caspase activities in various treatments were not due to changes in the level of caspase protein, which is evident from the Western blot analysis in Fig. 9. The lower caspase activity of DFMO-treated cells was not increased when supplemented with exogenous putrescine in the assay system (data not shown). These results indicate that polyamine depletion does not affect the level of caspase protein and does not directly modulate caspase activity, but that the intracellular polyamine pool is very important in the regulation of apoptosis.

As we have already pointed out in the introduction, high levels of polyamines induce apoptosis in a variety of cell types (34, 38, 43, 44, 57). Our data in Fig. 7 confirm these findings in that abnormally high putrescine levels caused apoptosis in IEC-6 cells. In addition, however, our results demonstrated for the first time that normal levels of polyamines are necessary for programmed cell death to occur within the usual time frame following an apoptotic stimulus. It is likely that polyamines are necessary for normal progression to apoptosis regardless of the stimulus. Camptothecin causes apoptosis by preventing the repair of damaged DNA, but we obtained identical results with a combination of TNF- α and cycloheximide (Fig. 5). TNF- α elicits an apoptotic response by binding to a physiological receptor.

Aberrant induction of ODC activity leads to malignant transformation or apoptosis, depending on the cell type and stimulus (44). Association of polyamines with DNA and RNA suggests that they play an important role in nucleic acid structure and function. Spermidine and spermine are able to condense DNA into a highly fluid structure, which is essential for biological function (41). The highest concentrations of polyamines are generally found in the G1 phase of the cell cycle (55) as a prerequisite in the cell's preparation for DNA synthesis. It has been suggested that the catenation of DNA by

topoisomerases requires the aggregation of DNA molecules by spermidine (25). Thus it is clear that polyamines play crucial roles in cellular organization. Transglutaminase induction by camptothecin has been reported as a possible cause of apoptosis in keratinocytes (28). Cell cycle regulators, like cdk2, also play crucial roles in the induction of apoptosis (18, 63). Polyamine depletion reduces tissue transglutaminase activity (61) and results in cell cycle arrest by induction of cdk inhibitory proteins in IEC-6 (47). Therefore, it is reasonable to believe that polyamine depletion associated changes in the physiological state of the cell may be responsible for a delay in apoptosis.

It appears from both the current results and our previous work (47) that polyamine depletion results in cell cycle arrest and the prevention of apoptosis by triggering active signal transduction pathways. This is an important concept that represents a change in the approach to understanding some functions of polyamines. Thus it is not only that some functions of the cell fail to occur in the absence of polyamines, but also that the absence of polyamines initiates events that result in changes in cell function. For instance, it is likely that the activation of p53, p21, and p27 in the absence of polyamines leads to the inhibition of cdk2 and produces cell cycle arrest (47). The cascade of events leading to the inhibition of apoptosis in polyamine-depleted cells remains to be elucidated.

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-16505, and by the Thomas A. Gerwin Endowment.

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Received 13 May 1999; accepted in final form 30 September 1999.

REFERENCES

1. Aoshiba, K., S. I. Rennard, and J. R. Spurzem. Cell-matrix and cell-cell interactions modulate apoptosis of bronchial epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 272: L28-L37, 1997.
2. Bates, R. C., A. Buret, D. F. van Helden, M. A. Horton, and G. F. Burns. Apoptosis induced by inhibition of intercellular contact. *J. Cell Biol.* 125: 403-415, 1994.
3. Beaulieu, J. F. Differential expression of the VLA family of integrins along the crypt-villus axis in the human small intestine. *J. Cell Sci.* 102: 427-436, 1992.
4. Boudreau, N., C. J. Simpson, Z. Werb, and M. J. Bissell. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* 267: 891-893, 1995.
5. Bradford, M. A. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Anal. Biochem.* 72: 224-254, 1976.
6. Brooks, W. H. Polyamine involvement in the cell cycle, apoptosis, and autoimmunity. *Med. Hypotheses* 44: 331-338, 1995.
7. Chen, X., L. J. Ko, L. Jayaraman, and C. Prives. p53 levels, functional domains, and DNA damage determine the effect of the apoptotic response of tumor cells. *Genes Dev.* 10: 2438-2451, 1996.
8. Cunningham, D. Setting a new standard irinotecan(campto) in the second-line therapy of colorectal cancer: final results of two phase III studies and implications for clinical practice. *Semin. Oncol.* 26, Suppl. 5: 1-5, 1999.
9. Cunningham, D., and B. Glimelius. A phase III study of irinotecan (CPT-11) vs. best supportive care in patients with metastatic colorectal cancer who failed 5 fluorouracil therapy. V301 study group. *Semin. Oncol.* 26, Suppl. 5: 6-12, 1999.

10. Davis, R. H., D. R. Morris, and P. Coffino. Sequestered end products and enzyme regulation: the case of ornithine decarboxylase. *Microbiol. Rev.* 56: 280-290, 1992.
11. Erhardt, P., K. J. Tomaselli, and G. M. Cooper. Identification of the MDM2 oncoprotein as a substrate for CPP32-like apoptotic proteases. *J. Biol. Chem.* 272: 15049-15052, 1997.
12. Farschon, D. M., C. Couture, T. Mustelin, and D. D. Newmeyer. Temporal phases in apoptosis defined by the actions of Src homology 2 domains, ceramide, Bcl-2, interleukin-1 β converting enzyme family proteases, and a dense membrane fraction. *J. Cell Biol.* 137: 1117-1125, 1997.
13. Frisch, S. M., and H. Francis. Disruption of epithelial cell matrix interactions induces apoptosis. *J. Cell Biol.* 124: 619-626, 1994.
14. Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119: 493-501, 1992.
15. Gervais, F. G., N. A. Thornberry, S. C. Ruffolo, D. W. Nicholson, and S. Roy. Caspases cleave focal adhesion kinase during apoptosis to generate a FRNK-like polypeptide. *J. Biol. Chem.* 273: 17102-17108, 1998.
16. Grassilli, E., M. A. Desiderio, E. Bellesia, P. Salomoni, F. Benatti, and C. Franceschi. Is polyamine decrease a common feature of apoptosis? Evidence from gamma rays- and heat-induced cell death. *Biochem. Biophys. Res. Commun.* 126: 708-714, 1995.
17. Grossmann, J., S. Mohr, E. G. Lapetina, C. Fiocchi, and A. D. Levin. Sequential and rapid activation of select caspases during apoptosis of normal intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 274: G1117-G1124, 1998.
18. Haleem, A., S. Takehiko, I. Kozieradzki, and J. M. Penninger. The cyclin-dependent kinase cdk2 regulates thymocyte apoptosis. *J. Exp. Med.* 189: 957-967, 1999.
19. Heby, O., and L. Presson. Molecular genetics of polyamine synthesis in eukaryotic cells. *Trends Biochem. Sci.* 15: 153-158, 1990.
20. Hermiston, M. L., and J. I. Gordon. In vivo analysis of cadherin function in the mouse intestinal epithelium: essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death. *J. Cell Biol.* 129: 489-506, 1995.
21. Hueber, A., P. Esser, K. Heimann, N. Kociok, S. Winter, and M. Weller. The topoisomerase I inhibitors, camptothecin and β -lapachone, induce apoptosis of human retinal pigment epithelial cells. *Exp. Eye Res.* 67: 525-530, 1998.
22. Kauffmann-Zeh, A., P. Rodriguez-Viciana, E. Ulrich, C. Gilbert, P. Coffey, J. Downward, and G. Evan. Suppression of c-Myc induced apoptosis by Ras signaling through PI(3)K and PKB. *Nature* 385: 544-548, 1997.
23. Kinoshita, T., T. Yokota, K. Arai, and A. Miyajima. Regulation of Bcl-2 expression by oncogenic Ras protein in hematopoietic cells. *Oncogene* 10: 2207-2210, 1995.
24. Kothakota S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T. J. McGarry, M. W. Kirschner, K. Koths, D. J. Kwiatkowski, and L. T. Williams. Caspase 3 generated fragment of gelsolin: effect of morphological change in apoptosis. *Science* 278: 294-298, 1997.
25. Krasnow, M. A., and Cozzarelli, N. R. Catenation of DNA rings by topoisomerases. Mechanism of control by spermine. *J. Biol. Chem.* 257: 2687-2693, 1982.
26. Kuida, K., J. A. Lippke, G. Ku, M. W. Harding, D. J. Livingston, M. S.-S. Su, and R. A. Flavell. Altered cytokine export and apoptosis in mice deficient in interleukin-1 β converting enzyme. *Science* 267: 2000-2003, 1995.
27. Lahti, J. M., J. Xiang, L. S. Heath, D. Campana, and V. J. Kidd. PITSLRE protein kinase activity is associated with apoptosis. *Mol. Cell Biol.* 15: 1-11, 1995.
28. Lin, X. R., D. I. Wilkinson, and E. M. Farber. Camptothecin induces differentiation, tissue transglutaminase and apoptosis in cultured keratinocytes. *Exp. Dermatol.* 7: 179-183, 1998.
29. Marton, L. J., and A. E. Pegg. Polyamines as targets for therapeutic intervention. *Annu. Rev. Pharmacol. Toxicol.* 35: 55-91, 1995.
30. Mashima, T., M. Naito, K. Noguchi, D. K. Miller, D. W. Nicholson, and T. Tsuruo. Actin cleavage by cyp-32/apopain during the development of apoptosis. *Oncogene* 14: 1007-1012, 1997.
31. McCormack, S. A., M. J. Viar, and L. R. Johnson. Migration of IEC-6 cells: a model for mucosal healing. *Am. J. Physiol. Gastrointest. Liver Physiol.* 263: G426-G435, 1992.
32. McCormack, S. A., M. J. Viar, and L. R. Johnson. Polyamines are necessary for cell migration by a small intestinal crypt cell line. *Am. J. Physiol. Gastrointest. Liver Physiol.* 264: G367-G374, 1993.
33. Meredith, J. E., Jr., B. Fazeli, and M. A. Schwartz. The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* 4: 953-961, 1993.
34. Mitchell, J. L., R. R. Diveley, Jr., A. Bareyal-Leyser, and J. L. Mitchell. Abnormal accumulation and toxicity of polyamines in a difluoromethylornithine-resistant HTC cell variant. *Biochim. Biophys. Acta* 1136: 136-142, 1992.
35. Miura, M., H. Zhu, R. Rotello, E. A. Hartwig, and J. Yuan. Induction of apoptosis in fibroblasts by IL-1 β -converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* 75: 653-660, 1993.
36. Moss, S. F., B. Agrawal, N. Arber, R. J. Guan, M. Krajewski, S. Krajewski, J. C. Reed, and P. R. Hoft. Increased intestinal Bak expression results in apoptosis. *Biochem. Biophys. Res. Commun.* 223: 199-203, 1996.
37. Nicholson, D. W., and N. A. Thornberry. Caspases: killer proteases. *Trends Biochem. Sci.* 22: 299-306, 1997.
38. Packham, G., and J. L. Cleveland. Ornithine decarboxylase is a mediator of c-Myc induced apoptosis. *Mol. Cell Biol.* 14: 5741-5747, 1994.
39. Pegg, A. E. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.* 48: 759-774, 1988.
40. Pegg, A. E., and H. G. Williams-Ashmann. In: *Polyamines in Biology and Medicine*, edited by D. R. Morris and L. J. Morton. New York: Marcel Dekker, 1981, p. 3-42.
41. Pelta J., F. Livolant, and J. Sikorav. DNA aggregation induced by polyamines and cobalthexamine. *J. Biol. Chem.* 271: 5656-5662, 1996.
42. Penning, L. C., R. G. Schipper, D. Vercammen, A. J. Verhofstad, T. Denecker, R. Beyaer, and P. Vandenabeele. Sensitization of TNF induced apoptosis with polyamine synthesis inhibitor in different human and murine tumor cell lines. *Cytokines* 10: 423-431, 1998.
43. Pouline, R., J. K. Coward, J. R. Lakanen, and A. E. Pegg. Enhancement of the spermidine uptake system and lethal effects of spermidine overaccumulation in ornithine decarboxylase overproducing L1210 cells under hypoosmotic stress. *J. Biol. Chem.* 268: 4690-4698, 1993.
44. Pouline, R., G. Pelletier, and A. E. Pegg. Induction of apoptosis by excessive polyamine accumulation in ornithine decarboxylase-overproducing L1210 cells. *Biochem. J.* 311: 723-727, 1995.
45. Quaroni, A., J. Wands, R. L. Trelstad, and K. J. Isselbacher. Epithelial cell culture from rat small intestine. *J. Cell Biol.* 80: 248-265, 1988.
46. Rak, J., Y. Mitsuhashi, V. Erdos, S.-N. Huang, J. Filmus, and R. S. Kerbel. Massive programmed cell death in intestinal epithelial cells induced by three dimensional growth conditions: suppression by mutant c-H-ras oncogene expression. *J. Cell Biol.* 131: 1587-1598, 1995.
47. Ray, R. M., B. J. Zimmerman, S. M. McCormack, T. B. Patel, and L. R. Johnson. Polyamine depletion arrests cell cycle and induces inhibitors p21 waf1/cip1, p27kip1, and p53 in IEC 6 cells. *Am. J. Physiol. Cell Physiol.* 276: C684-C691, 1999.
48. Re, F., A. Zanetti, M. Sironi, N. Polentarutti, L. Lanfranccone, E. Dejana, and F. Colotta. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. *J. Cell Biol.* 127: 537-546, 1994.
49. Sane, A. T., and R. Bertrand. Distinct steps in DNA fragmentation pathway during camptothecin induced apoptosis involved

- caspase-, benzyloxycarbonyl- and N-tosyl-L-phenylalanylchloromethyl ketone sensitive activities. *Cancer Res.* 58: 3066-3072, 1998.
50. Sarin, A., D. H. Adams, and P. A. Henkart. Protease inhibitors selectively block T cell receptor-triggered programmed cell death in a murine T cell hybridoma and activated peripheral T cells. *J. Exp. Med.* 278: 1693-1700, 1993.
 51. Scheving, L. A., W.-H. Jin, K.-M. Chong, W. Gardner, and F. O. Cope. Dying enterocytes downregulate signaling pathways converging on Ras: rescue by protease inhibition. *Am. J. Physiol. Cell Physiol.* 274: C1363-C1372, 1998.
 52. Sinicrope, F. A., S. B. Ruan, K. R. Cleary, L. C. Stephens, J. J. Lee, and B. Levin. Bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. *Cancer Res.* 55: 237-241, 1995.
 53. Strater, J., K. Koretz, A. R. Gunthert, and P. Moller. In situ detection of enterocytic apoptosis in normal colonic mucosa and in familial adenomatous polyposis. *Gut* 37: 819-825, 1995.
 54. Strater, J., U. Wedding, T. F. E. Barth, K. Koretz, C. Elsing, and P. Moller. Rapid onset of apoptosis in vitro follows disruption of $\beta 1$ integrin/matrix interactions in human colonic crypt cells. *Gastroenterology* 110: 1776-1784, 1996.
 55. Tabor, C. W., and Tabor, H. Polyamines. *Annu. Rev. Biochem.* 53: 749-790, 1984.
 56. Thornberry, N. A., H. G. Bull, J. R. Calaycay, K. T. Chapman, A. D. Howard, M. J. Kostuva, D. K. Miller, S. M. Molineau, J. R. Weidner, and S. Aunins. A novel heterodimeric cysteine protease is required for interleukin-1 β processing in monocytes. *Nature* 356: 768-774, 1992.
 57. Tobias, K. E., and C. Kahana. Exposure to ornithine results in excessive accumulation of putrescine and apoptotic cell death in ornithine decarboxylase overproducing mouse myeloma cells. *Cell Growth Differ.* 10: 1279-1285, 1995.
 58. Tomei, L. D., J. P. Shapiro, and F. O. Cope. Apoptosis in C3H/10T1/2 mouse embryonic cells: evidence for internucleosomal DNA modification in the absence of double stranded cleavage. *Proc. Natl. Acad. Sci. USA* 90: 853-857, 1993.
 59. Wang, J. Y., and L. R. Johnson. Role of ornithine decarboxylase in the repair process of gastric mucosal stress ulcers. *Am. J. Physiol. Gastrointest. Liver Physiol.* 258: G78-G85, 1990.
 60. Wang, J. Y., and L. R. Johnson. Luminal polyamines stimulate repair of gastric mucosal stress ulcers. *Am. J. Physiol. Gastrointest. Liver Physiol.* 259: G584-G592, 1990.
 61. Wang, J. Y., and L. R. Johnson. Role of transglutaminase and protein cross-linking in the repair of mucosal stress erosions. *Am. J. Physiol. Gastrointest. Liver Physiol.* 262: G818-G825, 1992.
 62. Wang, J. Y., M. Viar, P. Blanner, and L. R. Johnson. Expression of the ornithine decarboxylase gene in response to asparagine in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 271: G164-G171, 1996.
 63. Wang, J., and K. Walsh. Resistance to apoptosis conferred by cdk inhibitors during myocyte apoptosis. *Science* 273: 359-361, 1996.
 64. White, E. Life, death and the pursuit of apoptosis. *Genes Dev.* 10: 1-15, 1995.
 65. Xie, X., M. E. Tome, and E. W. Gerner. Loss of intracellular putrescine pool-size regulation induces apoptosis. *Exp. Cell Res.* 230: 386-392, 1997.
 66. Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 β converting enzyme. *Cell* 75: 641-652, 1993.

